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The main goal of this project is to elucidate the molecular mechanism of action of one of the Clostridial neurotoxins, tetanus toxin. The major approach has been to utilize cultured cells of neural origin, the rat pheochromocytoma cell line, PC12, to study the biochemistry of tetanus toxin action. We have found that the toxin is potent in inhibiting release of neurotransmitters from these cells. Significant progress has been made in identifying the mechanism. Results reveal that tetanus toxin inhibits the accumualtion of an important intracellular signalling molecule, cGMP. By the use of this information we have been able to reverse the toxic effects of tetanus on these cells by pharmacological agents that elevate levels of cGMP in PC12 cells, such as 8Br-cGMP and zaprinast. Current focus on the research includes studies on the reason why cGMP is depressed in tetanus infected cells. Successful completion of this project will provide useful therapeutic approaches for the treatment of a wide range of the lethal Clostridial infections.

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MECHANISM OF ACTION OF THE PRESYNAPTIC NEUROTOXIN, TETANUS TOXIN

ANNUAL REPORT

Terry B. Rogers, Ph.D

April 30, 1988

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University of Maryland School of Medicine
Baltimore, Maryland 21201

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FOREWORD

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals" prepared by the Committee on the Care and Use of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

Tetanus Toxin - Significance

Neurotoxins are invaluable tools in the study of neurotransmission (Ceccarelli & Clementi, 1979). For example, our understanding of the nicotinic acetylcholine receptor was greatly enhanced by studies using the snake venom toxin, α-bungarotoxin (Lee, 1972). Tetrodotoxin and saxitoxin were instrumental in characterizing the sodium channel.

Tetanus toxin, an example of a protein neurotoxin in a class of bacterial *Clostridial* toxins, is an important toxin that warrants investigation for several reasons. First, it is a potent inhibitor of neurotransmitter release. In fact, it is one of the most potent neurotoxins known to man (LD_{50} in rodents is 1 ng/kg) (Weilhoner, 1982). Its potency suggests that the toxin acts selectively at specific recognition sites in the central nervous system (CNS) that are critical for neuronal function. The potency of the toxin taken together with the fact that relatively molecules within a cells can evoke the response strongly suggest that tetanus toxin acts as an enzyme. In this way, it may be analogous to the well characterized cholera, pertussis and diphtheria toxins (Gill, 1976; Cassel & Pfeuffer, 1978; Gill & Meren, 1978; Uchida, 1983; Neville and Hudson, 1986).

Secondly, an important characteristic of tetanus toxin is that its site of action is presynaptic. Exposure of primary cultured neurons, brain slices, isolated neuromuscular preparations and synaptosomes to tetanus toxin results in inhibition of neurotransmitter release from presynaptic terminals (Collingridge et al., 1980; Bigalke et al., 1978; Pearce et al., 1983; Osborne & Bradford, 1973). Electrophysiological studies have demonstrated that tetanus decreases the spontaneous and evoked release of neurotransmitter while leaving postsynaptic membranes intact and responsive to agonists (Curtis &

DeGroat, 1968; Davies & Tongroach, 1979; Bergey et al., 1983). Very little is known about the molecular mechanism of action of tetanus toxin once it gains access to the presynaptic terminal. It is clear that it does not cause cell death or significant morphological damage (Simpson, 1986). The current view is that tetanus toxin interferes with a crucial biochemical step in the neurotransmitter release process. Since very little is known about the mechanism of neurosecretion, tetanus toxin is a valuable probe in gaining insights into this critical neural process. Thus studies on the molecular mechanism of action of tetanus toxin will not only provide important insights into the mechanism of action of the *Clostridial* neurotoxins, but should also yield valuable information on the mechanisms underlying neurotransmission.

In this research project we have decided to use cultured cell lines of neuronal origin to study the mechanism of action of tetanus toxin. The rat adrenal pheochromocytoma PC12 cell line (Greene & Tischler, 1982), offers a unique model system for such studies for several reasons. First, PC12 cells represent a homogeneous population of cells. Second, these cells have one of the most highly differentiated neurotransmitter release systems found in any cultured cell line and it has been well characterized (Green and Tischler, 1982). Third, nerve growth factor treated PC12 cultures, which are neuron-like in many ways, express high levels of complex gangliosides and show a high capacity to bind tetanus toxin (Walton *et al.*, 1988). Finally, during the course of the research sponsored in this contract we have recently demonstrated that PC12 cells are sensitive to tetanus toxin (Sandberg *et al.*, 1988a).

Thus the main focus of our research program has been to exploit this valuable cultured cell line, PC12, to examine the underlying biochemical

mechanisms responsible for the effects of the *Clostridial* neurotoxins on neurotransmission. As this report documents, several important discoveries have been made during the past year which document that our experimental approach has been a productive one.

Results from the Principal Investigator's Laboratory During the Past Year

The main focus of the research during the past year has been to further exploit the PC12 cultured cell system to characterize the molecular mechanisms of tetanus toxin inhibition of neuronal cell function. As we have recently reported (Sandberg *et al.*, 1988a, Walton *et al.*,1988) PC12 cells that are treated with nerve growth factor (NGF) are sensitive to tetanus toxin; that is, tetanus toxin is a potent inhibitor of the release of acetylcholine (ACh) from these cultures. The main progress during the past year of this research program has been to utulize this cultured cell system to characterize the steps in the intoxication pathway and to identify the potential site of action of the toxin.

Effects of Culturing Conditions of PC12 Cells on the Senstivity to Tetanus Toxin. PC12 cells will differentiate in a variety of ways in response to the culturing conditions. We have recently demonstrated that complex ganglioside and tetanus toxin receptors are expressed at rather low levels in undifferentiated cells and increased significasntly in NGF-treated cultures (Walton *et al.*, 1988). THerefore we have initiated and completed a detailed to study to examine the effects of differentiation of PC12 cells on their sensitivty to tetanus toxin. The main goal of this part of the program has been establish the optimal conditions for further studies.

PC12 cells were cultured under conditions known to stimulate distinct

forms of differentiation: nondifferentiated, low density for 7 days (SPARSE); glucocorticoid treatment, in the presence of dexamethasone for 14 days (DEX); NGF for 14 days (NGF); autodifferentiated, high density for 7 days (DENSE). As shown in Fig. 1, the culturing conditions had a marked effect on the sensitivity of the cells to tetanus toxin. In these experiments [^{3}H]ACh release from NGF-treated cells was inhibited by 81% whereas cells grown under any of the other conditions were insensitive to tetanus toxin. There was a larger evoked release of [^{3}H]ACh from NGF-treated cultures which can be explained, in part, by the 8-fold higher levels of choline acetyltransferase (CAT) expressed in these cells. CAT levels (in pmol ACh/min/mg protein) were: sparse, 140 $\pm$ 12; DEX-treated, 156 $\pm$ 16; dense, 802 $\pm$ 69; and NGF-treated, 988 $\pm$ 86. It is noteworthy that densely grown cells, which differentiate to express CAT at elevated levels, and do show a significant evoked release of [^{3}H]ACh, are completely insensitive to the toxin.

Closer examination of the development of sensitivity of NGF-treated cultures revealed that PC12 cells become sensitive to tetanus toxin only after culturing in NGF for 8 days or longer (Fig. 2). Day 6 cells were particularly poor at releasing ACh. The characteristics of [^{3}H]ACh release from day 3 cells were similar to that observed in densely grown cells (Fig. 1). This may reflect the fact that day-3 cells may be more similar to dense cells since day-0 cells were subcultured from confluent flasks. Taken together, these results demonstrate that the inhibitory effects of tetanus toxin on [^{3}H]ACh release are observed only in cultures that are grown for extended periods in the presence of NGF.

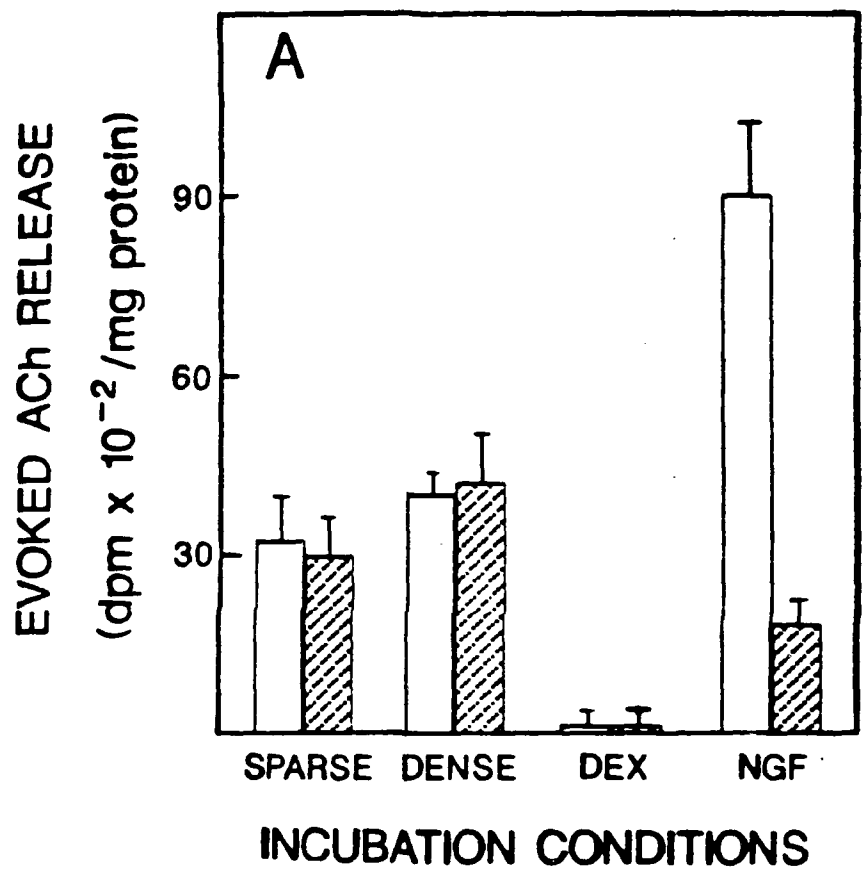

Figure 1. Effect of tetanus toxin on veratridine-evoked acetylcholine release from PC12 cells grown under various differentiation conditions. Veratridine-evoked [^{3}H]ACh release was measured from PC12 cells that had been preincubated with [^{3}H]Ch for 18 hr. Evoked [^{3}H]ACh release was measured in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 16-18 h incubation at 37°C) from PC12 cells grown under the following conditions: 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 1 x 10^{-6} M dexamethasone (DEX); 14 days at 5 x 10^4 cells/10 cm^2, in the presence of 100 ng/ml nerve growth factor (NGF); 7 days, at high density (5 x 10^5 cells/ 10 cm^2) (Dense); or at low density (5 x 10^4 cells/ 10 cm^2) (Sparse). The results are the means of 2-3 experiments each performed in sextuplet ± s.e.m.

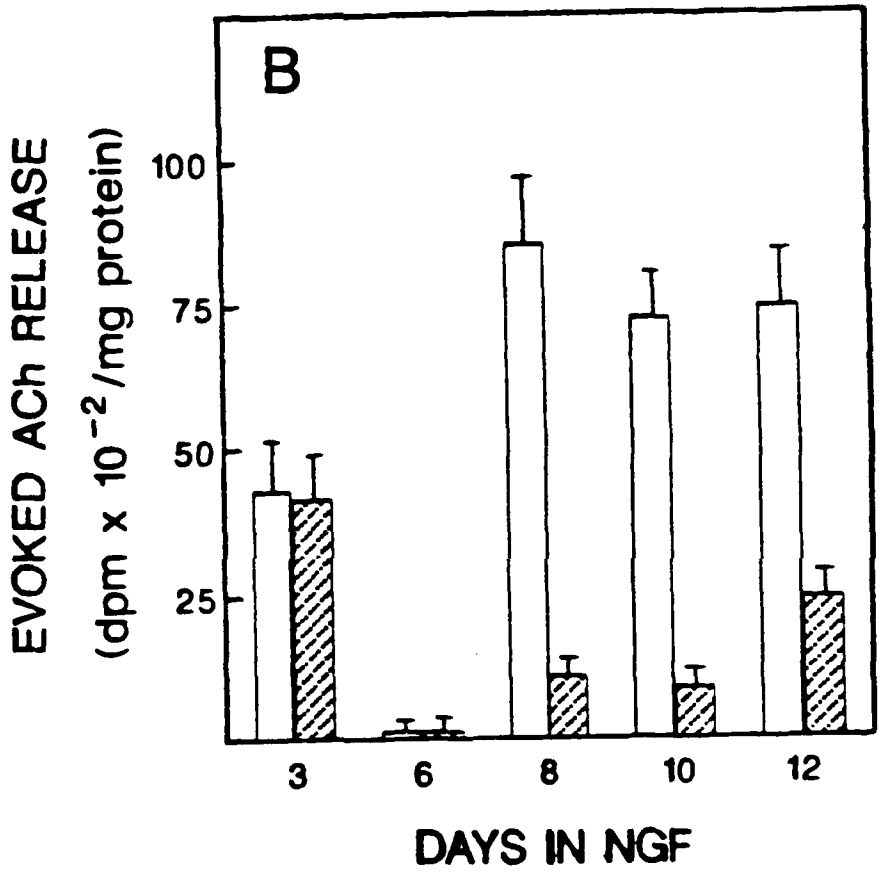

Figure 2. The effect of tetanus toxin on veratridine-evoked acetylcholine release from PC12 cells as a function of days in NGF. Evoked [^{3}H]ACh release was measured as a function of culture days in NGF (100 ng/ml) in the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM; 16-18 h incubation at 37°C). The results are the means of 2-3 experiments each performed in sextuplet ± s.e.m.

Time Course for the Development of the Effects of Tetanus Toxin on PC12 Cells. We have already reported that the effects of tetanus toxin on PC12 cells were time-dependent (Sandberg *et al.*, 1988a). As shown in Fig. 3, in time course studies using a maximal dose of toxin (10 nM), there was no effect on ACh release before 1.5 hr. After this lag period, the inhibitory effects on release rapidly developed during the following 1.5 hr. As shown in the semilog plot in Fig. 3B, the rate of onset during this period can be described as a first order process.

This characteristic lag phase observed for the action of tetanus toxin could be due to a slow rate of toxin binding or could result from rate limiting events that occur after initial toxin-cell interactions. In order to begin to evaluate this process, time-course studies were performed in which the binding of ^{125}I-tetanus toxin and the inhibitory responses were measured in parallel culture dishes. PC12 cells were incubated with 0.1nM, 1 nM, and 10 nM ^{125}I-tetanus toxin. At the indicated times, the amount of ^{125}I-tetanus toxin bound was determined and the [^{3}H]ACh release activity was assayed in cultures that had been incubated in parallel with unlabeled toxin (Fig. 4). Tetanus toxin was found to be very potent in inhibiting [^{3}H]ACh release. As shown in Fig. 4, doses of tetanus toxin in the range of 0.1-10 nM produced maximal inhibition, which was 70% in these experiments. Threshold doses occurred at 10 pM, in which 48 hr incubations produced variable (10-20%) inhibition (data not shown). However, the length of the lag phase and the rate of the development of the toxic effects were inversely related to toxin concentration. The lag phase ranged from 1 hr at 10 nM to 8 hr at 0.1 nM toxin. The maximal toxin effects were observed by 2 hr at 10 nM tetanus toxin (Fig. 4C) and required 20 hr to be seen at 0.1 nM toxin (Fig. 4A). As shown

In Fig. 4B, incubations with 1 nM toxin showed intermediate values.

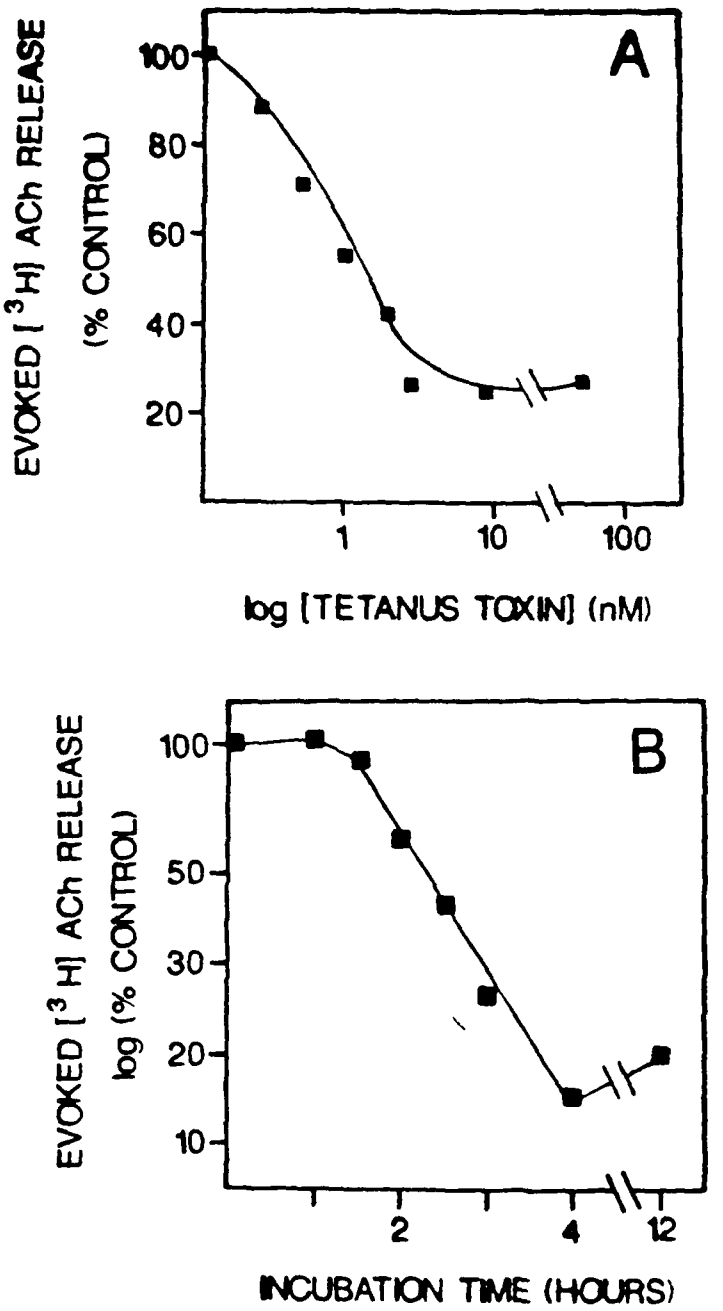

Fig. 3. Dose-response and time course for the tetanus toxin-induced inhibition of [^{3}H]ACh release from PC12 cells. Panel A, NGF-treated PC12 cells were incubated with increasing doses of tetanus toxin for 3 hr. Veratridine-evoked [^{3}H]ACh release was measured as described in Fig. 1A and "Experimental Procedures". Panel B shows results in which PC12 cells were incubated with 10 nM tetanus toxin. At various time points the amount of evoked [^{3}H]ACh release was measured as described in Fig. 1a and "Methods". The results are displayed as a semilogarithmic plot.

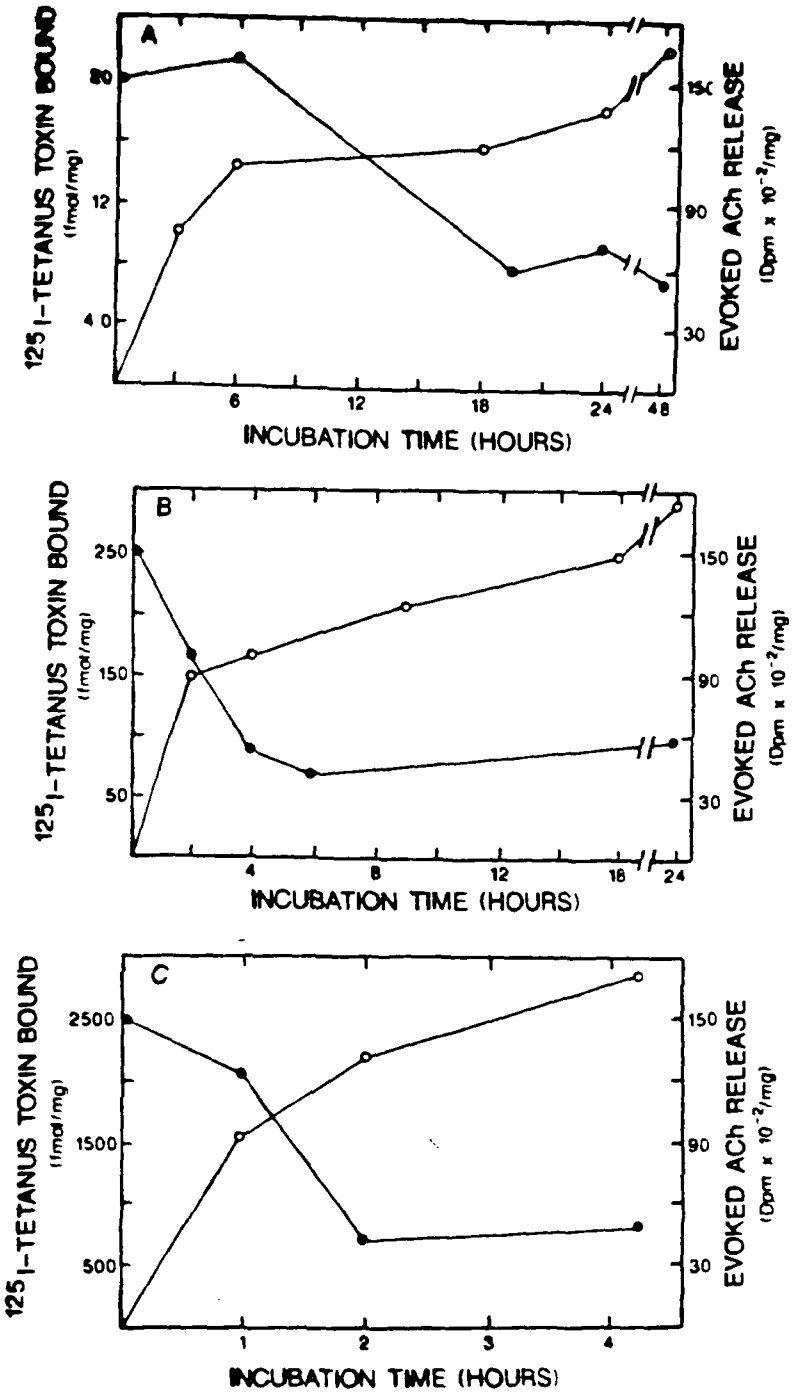

Figure 4. Comparison of tetanus toxin binding and inhibition of [^{3}H]ACh release from PC12 cells. Cultures of PC12 cells were incubated in parallel with 0.1 nM (Panel A), 1 nM (Panel B) or 10 nM (Panel C) ^{125}I-tetanus toxin or unlabeled tetanus toxin in culture medium without horse serum. At various time points the level of veratridine-evoked [^{3}H]ACh release ( ) was determined in the cultures that were incubated with unlabeled toxin and level of cell-associated toxin was measured in the cultures that had been incubated with ^{125}I-tetanus toxin ( ). The results are the means of sextuplet cultures with a variation of 10% or less.

Direct comparison of the time course of the onset of the toxic effects with the level of ^{125}I-tetanus toxin binding to PC12 cells revealed that a strict relationship between cell-associated toxin levels and inhibition of release did not exist (Fig. 4). For example, in the 0.1 nM ^{125}I-tetanus toxin experiments (Fig. 4A), toxin binding reached a plateau within 6 hr which was stable for at least 24 hr. However, the inhibitory effects developed only after a 6 hr lag time. Further, in 10 nM ^{125}I-tetanus toxin 1 hr incubations, 1500 fmol of toxin/mg of protein was cell-associated while only a 20% inhibition of [^{3}H]ACh release was observed (Fig. 4C). In contrast, when PC12 cells were incubated with 0.1 nM toxin for 18 hr, only 16 fmol of ^{125}I-tetanus toxin/mg of protein were bound, yet [^{3}H]ACh release was inhibited by 65% (Fig 4A). Taken together, these data indicate that the lag phase in the action of toxin is not the result of the slow rate of tetanus toxin binding to PC12 cells. Further, these results underscore the complexity of the intoxication process. It is likely that other intermediate steps that follow initial binding are necessary in order for the inhibitory effects of tetanus toxin to be expressed.

Internalization of Tetanus Toxin. We have recently reported that PC12 cells can rapidly internalize tetanus toxin that has been bound to the cell surface (Sandberg *et al.*, 1988b). These studies were performed in incubations where low ionic stength buffers were utilized in order to obtain a sufficient binding signal with 0.2 nM ^{125}I-tetanus toxin. However it was important to determine if tetanus toxin could be internalized by PC12 cells under conditions identical to those that were used in the functional studies. Accordingly, PC12 cells were incubated with 0.1 nM ^{125}I-tetanus toxin in DMEM, 0.5% BSA and toxin internalization was assessed in experiemtns in which

pronase was used to remove noninternalized toxin. As shown in Table 1, in cultures incubated with toxin for 3 hr at 0°C, very little internalization occurred. However in the 37°C incubations , significant internalization was observed. Thus at low toxin doses, under conditions identical to those used in the functional studies, about 400 molecules of ^{125}I-tetanus toxin/cell were internalized.

The results from the time course studies revealed that the 400 molecules are internalized during the lag phase before the toxic responses are observed. We decided to investigate the possibility that if 400 molecules of tetanus toxin were internalized, this was a sufficent quantity of internalized toxin to ultimately lead to inhibition of ACh release following the observed lag phase. In order to examine this hypothesis "protease-pulse" experiments were performed. PC12 cells were incubated with unlabeled tetanus toxin (0.1 nM) for 3 hr at either 0°C or 37°C. These cultures were treated with pronase, as

TABLE 1

Correlation of ^{125}I-Tetanus Toxin Binding

With Internalization and Inhibition of [^{3}H]ACh Release

Incubation Temperature	Cell Associated ^{125}I-Tetanus Toxin					Evoked [^{3}H]ACh Release (dpm x10^{-2}/ mg protein)	
	Total		After Pronase		Molecules Internalized	Control	Toxin
	fmol/mg	mol/cell	fmol/mg	mol/cell			
0°C	2.4 ± 0.4	724	0.2 ± 0.2	660	64	35 ± 6	30 ± 6
37°C	2.7 ± 0.3	807	1.4 ± 0.2	386	421	55 ± 7	11 ± 3

NGF-treated PC12 cells (5 x 10^{5} cells / 10cm^2) were incubated with ^{125}I-tetanus toxin (0.1 nM) for 3 hr at 37°C in DMEM, 0.5% BSA. After the incubation, the cells were extensively washed and the total cell-associated ^{125}I-tetanus toxin and fraction of bound ^{125}I-toxin that was pronase resistant was determined. Due to the low levels of binding under these experimental conditions, the data is expressed as the means of experiments performed in triplicate in which each data point is the combination of four 10 cm^2 wells ± s.e.m. In parallel experiments cultures were incubated with unlabeled tetanus toxin (0.1 nM) and treated with pronase. The cells were washed to remove unbound tetanus toxin and pronase and the cultures were incubated for another 18 hr with [^{3}H]Ch before veratridine-evoked [^{3}H]ACh release was measured as described in "Experimental Procedures" and Fig. 1A. The results are expressed as the means of sextuplet cultures ± s.e.m. There was a reduction in the levels of [^{3}H]ACh release in these experiments, most likely because harsh experimental conditions (temperature changes and protease treatment).

above, to remove surface-bound toxin. The cells, after complete washing, were returned to the incubator for 24 hr to preload with [^{3}H]Ch before ACh release activity was measured. As shown in Table 1, [^{3}H]ACh release was inhibited by 80% in cultures that had been "pulsed" with 0.1 nM toxin at 37°C whereas release was inhibited by only 14% in cultures that had been "pulsed" with toxin at 0°C. These results support the view that sufficient toxin molecules were internalized in 3 hr incubations with 0.1 nM tetanus toxin to ultimately give rise to inhibition of ACh release 24 hr later.

We have performed other studies in order to examine the role of internalization in the intoxication pathway. It was possible to take advantage of the observations that at 0°C internalization of tetanus toxin is blocked. Further, results form our laboratory reveal that is was possible to measure Ca^{2+}-dependent, voltage-dependent release of ACh from PC12 cells. By exploiting these two observations, we performed experiemnts to examine the effect of incubation temperature on the sensitivity of PC12 cells to tetanus toxin. As shown in Table 2, when PC12 cells were incubated with toxin at 0°C, there was no inhibtion of ACh release. However, when cells were incubated with toxin at 37°C, ACh release was inhibited when measured at either 0°C or 37°C. When similar experiments were performed using a barium-evoked release protocol, similar results were obtained (Fig. 5). Thus the sensitivity of the cells to tetanus toxin is dependent upon the incubation temperature and the temperture at which the release assay was performed.

Table 2

Effect of Incubation Temperature on the Potency of Tetanus Toxin to Block Veratridine-Evoked Acetylcholine Release from PC12 Cells

Preincubation Temperature (°C)	ACh Release Assay Temperature (°C)	[Calcium]	Evoked ACh Release (dpm $\times 10^{-2}$ /mg protein) Control	Toxin
37	37	0	5 ± 0.7	ND[a]
37	37	2 mM	68 ± 6.1	ND[a]
0	0	0	4 ± 0.8	ND[a]
0	0	2 mM	32 ± 3.7	29 ± 2.3
37	0	2 mM	39 ± 4.0	11 ± 0.9

PC12 cells were preincubated with 10 nM tetanus toxin at either 37°C or 0°C as indicated by the preincubation temperature. The results are the means of sextuplet cultures ± s.e.m.

[a] Not determined in these experiments

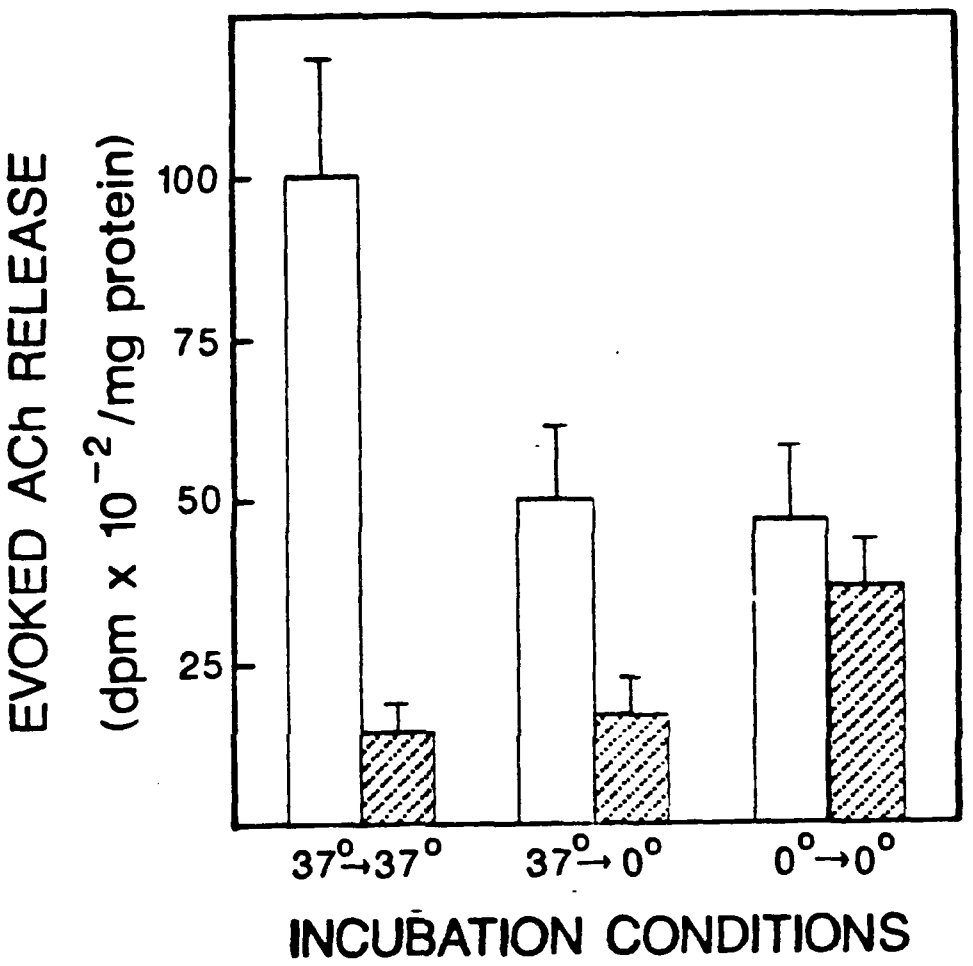

Figure 5. Effect of temperature on the potency of tetanus toxin to inhibit Ba^{2+}-evoked [^{3}H]ACh release from PC12 cells. Ba^{2+}-evoked [^{3}H]ACh release was defined as the total [^{3}H]ACh released in the presence of barium depolarizing buffer (Release buffer in which barium replaced calcium) minus the levels of [^{3}H]ACh released spontaneously ([^{3}H]ACh release measured in the presence of Release buffer). Evoked [^{3}H]ACh release was measured in cultures that had been incubated the presence (hatched bars) and absence (open bars) of tetanus toxin (10 nM, 3 hr). Incubation temperatures were as follows: 37-37°C, 37°C toxin incubation, 37°C release incubation; 37-0°C, 37°C toxin incubation, 0°C release incubation; and 0-0°C, 0°C toxin incubation, 0°C release incubation. The results are the means of sextuplet cultures ± s.e.m.

Role of cGMP in the Action of Tetanus Toxin. We reported in the last annual report that analogues of cGMP were able to reverse the toxic effects of tetanus on ACh release on PC12 cells. In order to further characterize the role of cGMP in the mechanism of tetanus toxin, the effects of inhibitors of cGMP degrading phosphodiesterases were examined. Fig. 6, Panel A shows the effects of phosphodiesterase inhibitors on cGMP levels in normal (open bars) and toxin-treated PC12 cells (hatched bars). In the control experiments, 10 nM tetanus toxin decreased the depolarization-evoked accumulation of cGMP by 75%.. Treatment of the cells with 100 μM IBMX resulted in an increase in the cGMP accumulation in normal cells (by 50%) and a slight increase in cGMP accumulation relative to the IBMX-free cultures. However, another phosphodiesterase inhibitor, zaprinast, which has been reported to be specific for a cGMP-degrading phosphodiesterase (Lugnier *et al.*, 1986) had a significant effect on cGMP accumulation in normal cells. Zaprinast (1 μM) increased evoked cGMP accumulation by nearly 100%. Zaprinast also completely reversed the inhibitory effects of tetanus toxin on this cGMP accumulation (Figure 6, Panel A).

If the inhibition of cGMP accumulation is causally related to the mechanism of action of tetanus toxin, then agents which restore the stimulus evoked increase in cGMP should also restore neurotransmitter release. This hypothesis was confirmed in results presented in Figure 6, panel B. In control experiments, tetanus toxin treatment resulted in the inhibition of ACh release by 80%. Application of IBMX resulted in a slight increase in the amount of ACh released from toxin-treated cells. However, zaprinast completely reversed the inhibitory effects of tetanus toxin on ACh release from PC12 cells. In these experiments PC12 cultures had been preincubated

with 10 nM toxin for 16 hr and were then exposed to 1 μm zaprinast for 2 min just prior to performing the release assay. Thus, we have discovered another agent which is potent in reversing the effects of tetanus toxin once they been established in neural cells.

Comparison of the results from Panel A and Panel B reveal that there is a remarkable correlation between the ability of phosphodiesterase inhibitors to restore cGMP accumulation and ACh release in tetanus toxin-treated PC12 cells. Taken together, these striking results support the hypothesis that part of the mechanism of action of tetanus toxin is due to a toxin-mediated activation of a cGMP-specific phosphodiesterase in neural tissues.

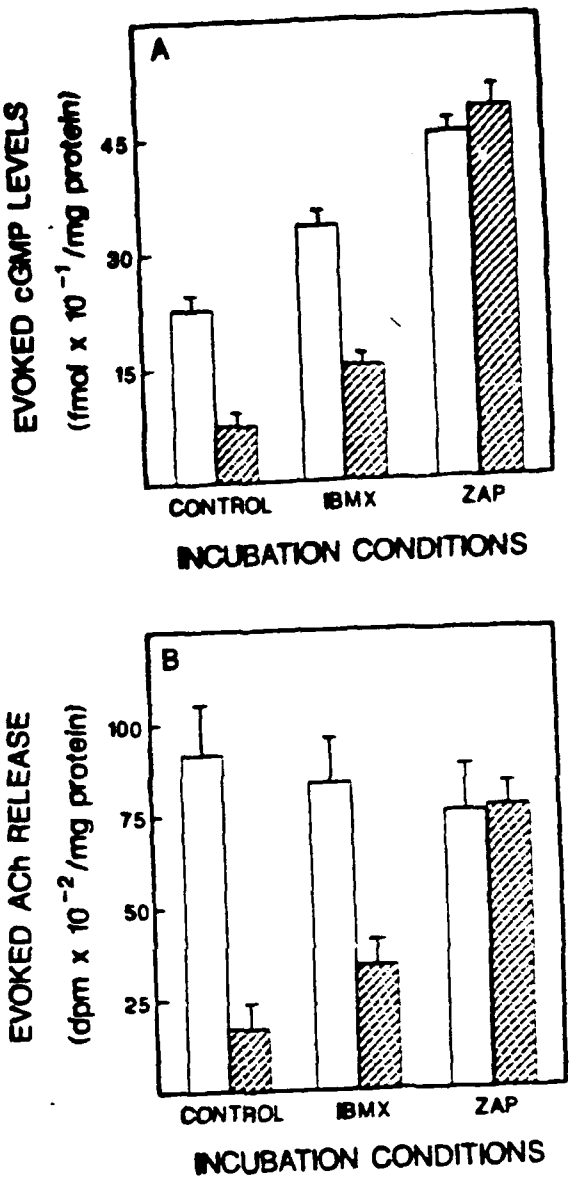

Figure 6. Reversibility of Tetanus Toxin Action on PC12 Cells by Phosphodiesterase Inhibitors. In Panel A evoked levels of cGMP were measured on cells preincubated (16-18 h at 37°C) with (hatched bars) and without (open bars) 10 nM tetanus toxin. Panel B shows the results where evoked [^{3}H]ACh release was measured on these same cells, simultaneously. Evoked cGMP levels and evoked [3H]ACh release levels are defined as total stimulated levels minus basal levels. Basal levels were measured in the presence of "Control release buffer" and stimulated levels were measured in the presence of "Depolarizing release buffer" (Control release buffer supplemented with 200 µM veratridine) after incubation in release buffer for 45 sec at 37oC. Before cells were exposed to release buffers, they were washed twice (1 min each wash) with the following buffers: Control, DMEM alone; IBMX, DMEM supplemented with 100 µM IBMX; ZAP, DMEM supplemented with 1 µM zaprinast. These results are the means of 2-3 experiments each performed in sextuplet ± s.e.m.

CONCLUSIONS

During the early phase of the research conducted under this contract we were successful in establishing a cultured cell line of neural origin, PC12 cells, as a useflu model system to study the mechanism of action of tetanus toxin at the molecular level. During the past year significant progress has been made in exploiting this cellular system and the results have revealed important observations concerning the pathway of intoxication and the involvement of second messengers in the mechanism of action of tetanus.

Studies on the effects of differentiation on the sensitivity of PC12 cells to tetanus toxin have yielded some important results. Only nerve growth factor-differentiated PC12 cells are sensitive to tetanus toxin. It is of interest to note that NGF-treated cultures are more neuron-like (Green and Tischler, 1982) and express higher levels of complex gangliosides and tetanus binding (Walton *et al.*, 1988). Thus the differentiation state was a criycal factor in determining tetanus sensitivity. These results may explain why many other laboratories have not been successful in observing the effects of tetanus on PC12 cells.

Important prgress has been made during the past year in elucidating the intoxication pathway of tetanus toxin in PC12 cells. The opportunity to exploit a system comprized of a homogeneous population of cultured cells was avalable to us. This allowed for studies that correlated molecular events with cellular responses to tetanus toxin at a level not previously possible. Time course studies revealed that a lag phase existed which was inversely related to toxin concentration, before the inhibitory response (which could be described as a first order process) was observed. A latent period has been previously observed in primary cultured spinal cord neurons and the

neuromuscular junction following tetanus treatment (Schmitt *et al.*, 1981; Bergey *et al.*, 1983). It is interesting to note that other protein toxins, such as diphtheria, abrin and ricin show qualitatively similar properties in their onset kinetics (Elsworthy and Neville, 1984; Neville and Hudson, 1986). These results have been widely interpreted to mean that there is an intracellular processing step preceding toxin action in these systems (see Neville and Hudson, 1986 for a review). A similar process is likely to occur with tetanus toxin in PC12 cells.

Further studies revealed that the intoxication pathway could be biochemically dissected into several definable steps. Results reported here demonstrate that there is a lack of coupling between tetanus toxin binding to the surface of PC12 cells and the response. Further, this binding is followed by a rapid temperature dependent internalization process with a half life of 2-4 min. Although these results are not conclusive, they do support the idea that the rate limiting step follows the internalization process in PC12 cells. Results from a "protease-pulse" studies provide further support for this hypothesis. These studies showed that it was possible to internalize a relatively low amounts of toxin in 3 hr without inhibiting release. However, upon further incubation in the absence of additional tetanus toxin the inhibitory effects did develop. These results are interpreted to mean that the processing and delivery events are rate limiting in the intoxication pathway. This conclusion underscores the importance of additional processing events in the pathway. In other incubation experiments, by the use of low temperature, it was possible to restrict cell-associated toxin to the cell surface. The lack of inhibition of ACh release in these low temperature incubations combined with the protease-pulse results provides evidence that

internalization is a necessary event in the intoxication pathway. However, it is not clear from these studies if other temperature-dependent processing steps follow internalization. An important goal in the future will be to further delineate specific steps in the intoxication pathway in order to answer this question.

A most significant development during the past year has been further accumulating experimental evidence that implicates cGMP metabolism with the mechanism of action of tetanus toxin. An important discovery that further supports this hypothesis has been the observation that zaprinast, a potent and specific inhibitor of cGMP degrading phosphdiesterase (Lugnier *et al.*, 1986), reverses the inhibitory effects of tetanus toxin. Thus the accumulating evidence from our laboratory strongly implicates tetanus toxin action with cGMP: (1) There was an excellent correlation between the ability of tetanus to block ACh release and to inhibit evoked levels of cGMP in intact cells. (2) Agents that restored cGMP levels by independent mechanisms, either inhibition of phosphdiesterase or addition of cGMP analogues directly, restored the release of ACh in toxin treated cells.

Evidence gathered in recent years has suggested that cGMP may be involved in regulating nervous system function (Ferrendelli, 1978; Paupardin-Tritsch *et al.*, 1986). Several reports have demonstrated an elevation of cGMP levels in response to neurotransmitters and to depolarizing agents in neural tissue preparations (Ohga and Daly, 1977; Ferrendelli *et al.*, 1973). However, it has not been possible to assess the precise role, if any, for cGMP in neurotransmitter release. The recent discoveries associating the action of tetanus toxin with cGMP suggest that tetanus will be an excellent probe for such studies. A major focus in the coming year will be to further clarify

these interesting proposals.

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PERSONNEL INVOLVED IN CONTRACT WORK

1. Kathryn Sandberg, Graduate Student, 80% Time, 04/01/87-06/31/87

2. Cathy Berry, Research Assistant, 100% time, 04/01/87-08/31/87

3. Andrea Grandin, MS, Research Assistant, 100% time, 10/01/87-present.

4. David Evans, PhD, Postdoctoral Research Associate, 100% Time, 11/01/87-present.

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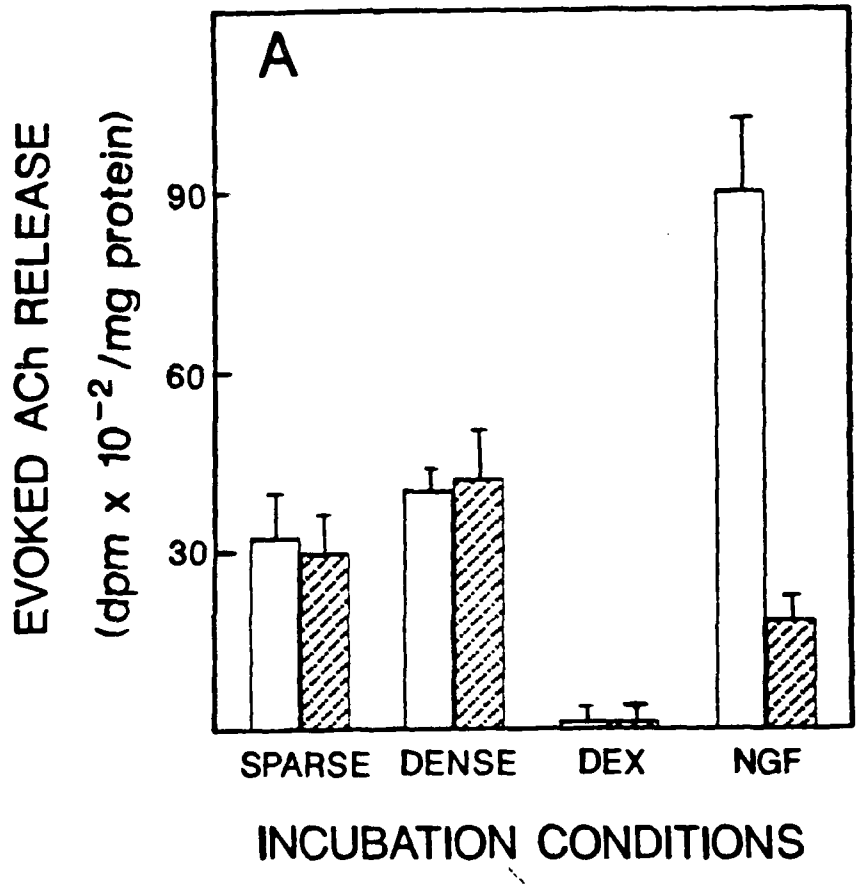
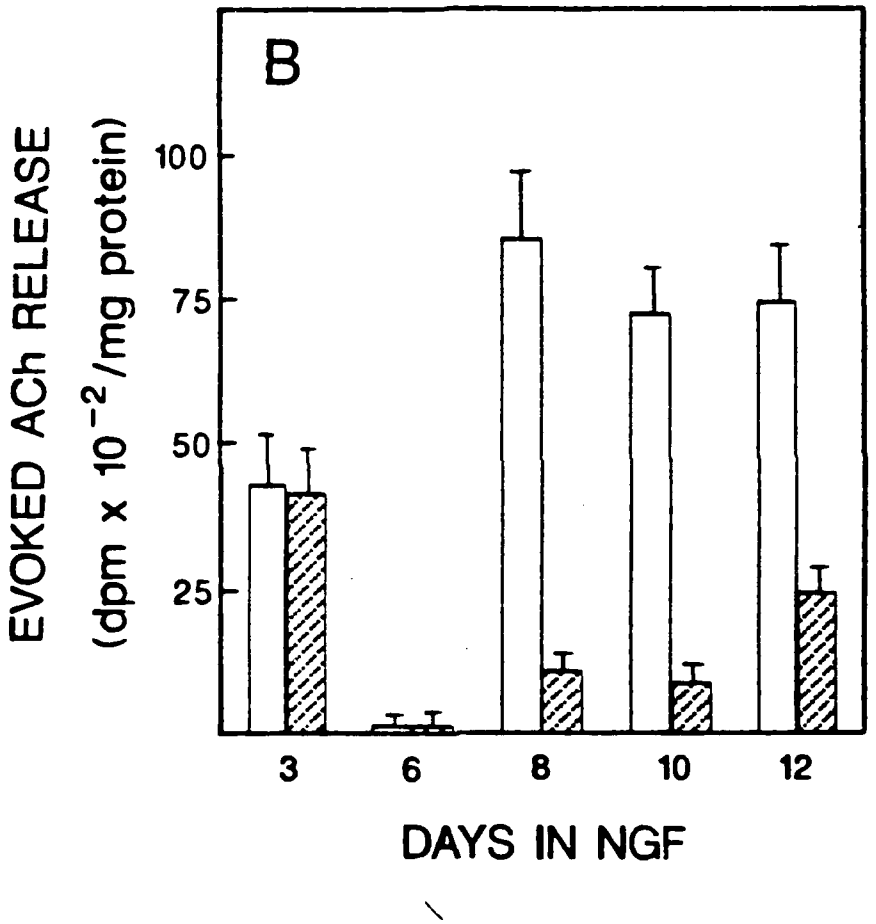
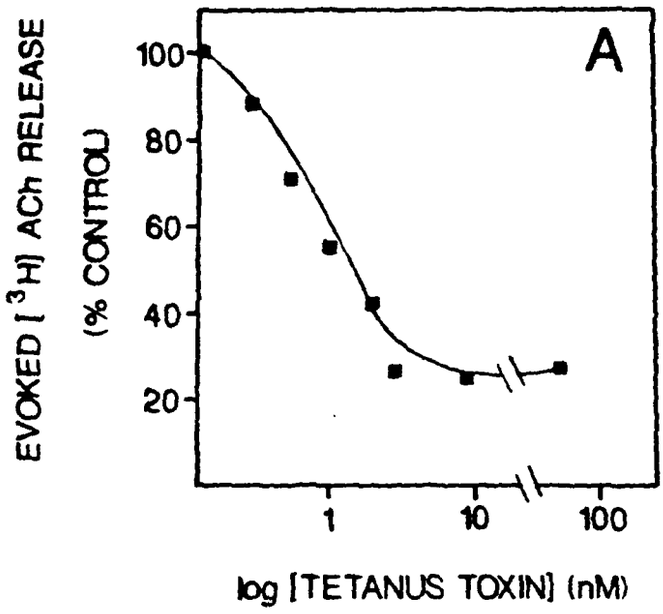
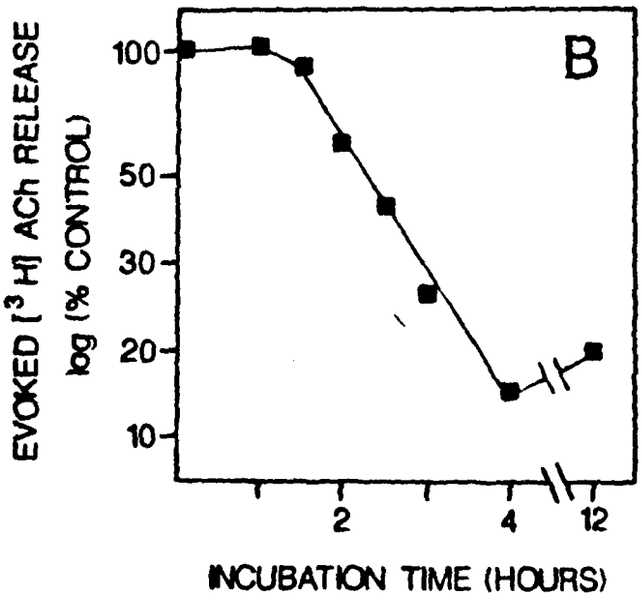
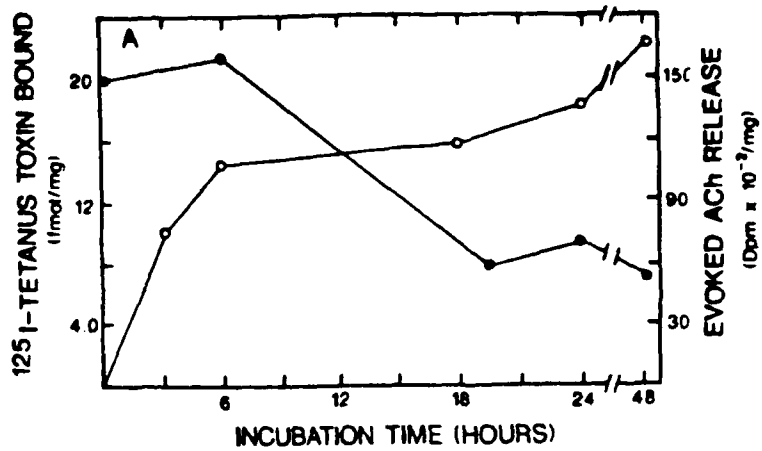
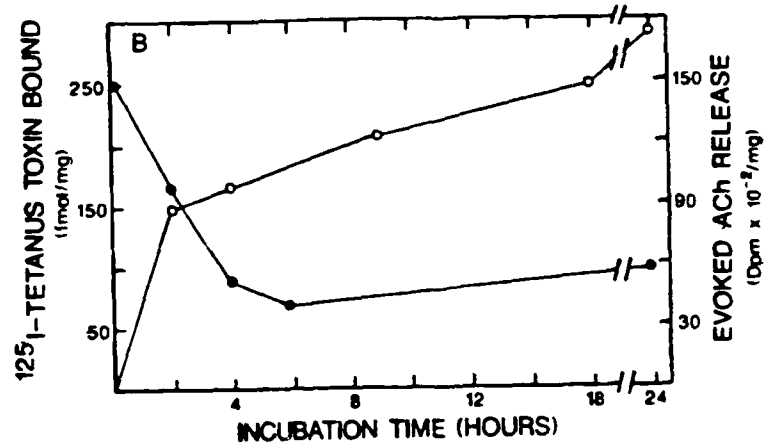

A
EVOKED ACh RELEASE
(dpm x 10⁻² /mg protein)
90
60
30
SPARSE
DENSE
DEX
NGF
INCUBATION CONDITIONS

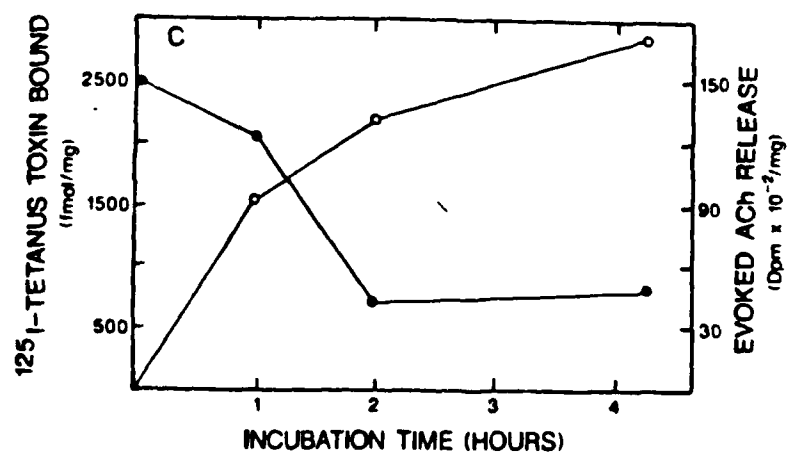

B
EVOKED ACh RELEASE
(dpm x 10⁻²/mg protein)
100
75
50
25
3
6
8
10
12
DAYS IN NGF

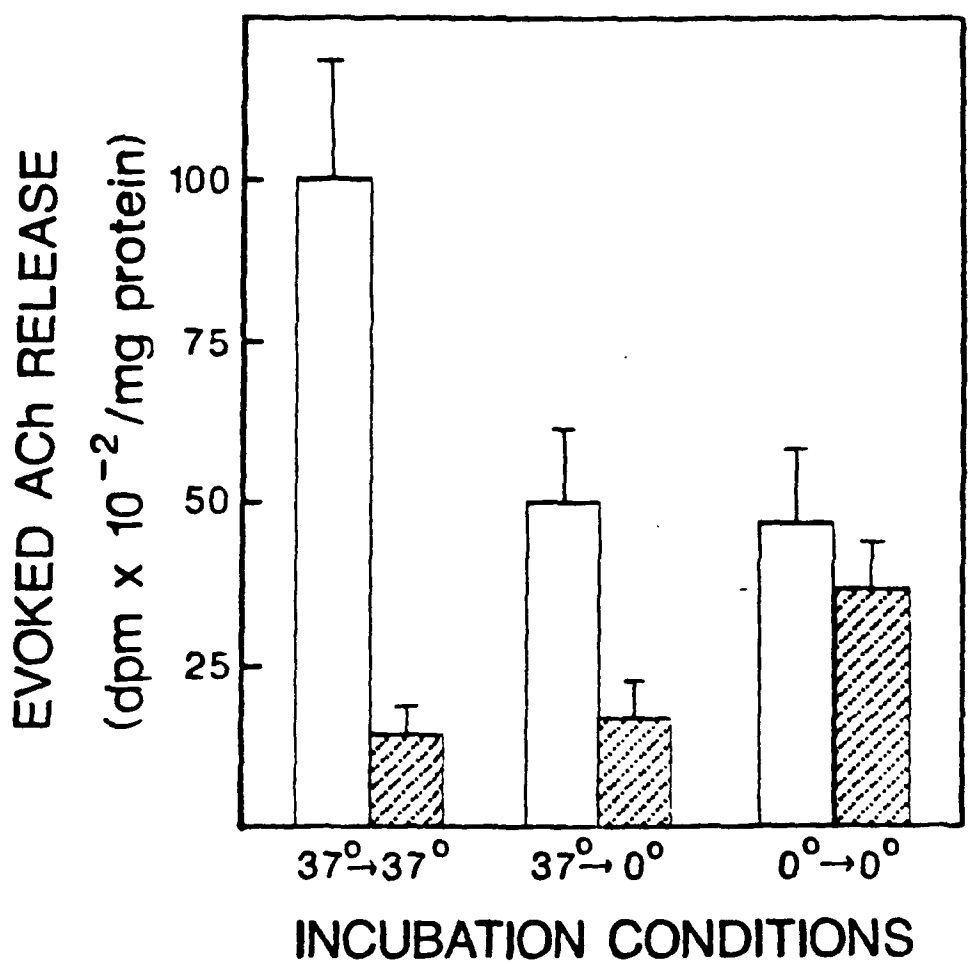

A
EVOKED [^{3}H] ACh RELEASE
(% CONTROL)
100
80
60
40
20
1
10
100
log [TETANUS TOXIN] (nM)

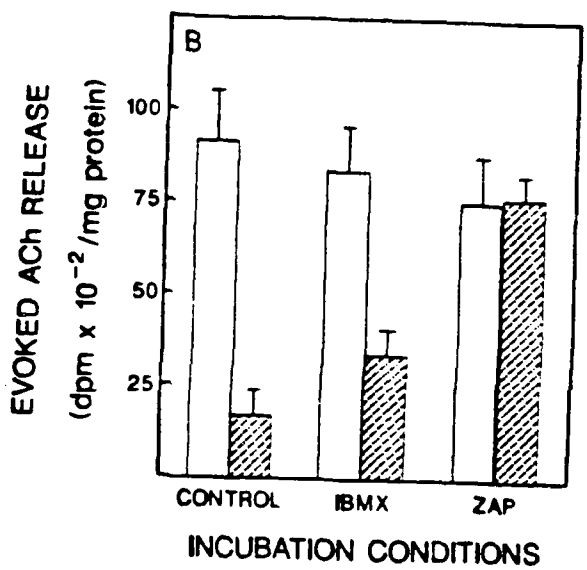

B
EVOKED [^{3}H] ACh RELEASE
log (% CONTROL)
100
50
30
20
10
2
4
12
INCUBATION TIME (HOURS)

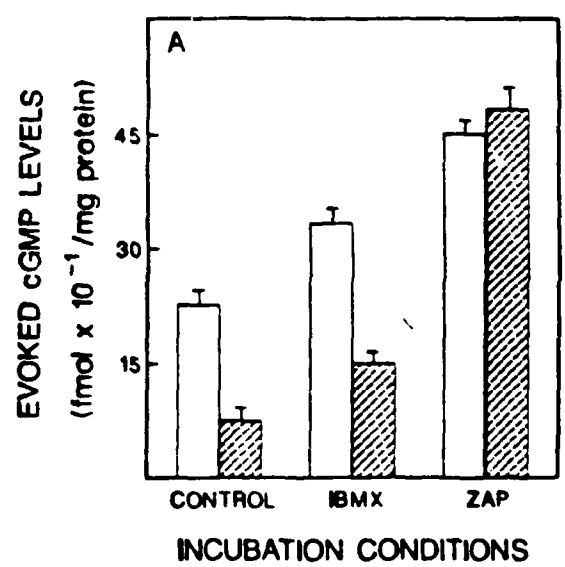

A
^{125}I-TETANUS TOXIN BOUND
(fmol/mg)
20
12
4.0
150
90
30
EVOKED ACh RELEASE
(Dpm x 10^{-3}/mg)
6
12
18
24
48
INCUBATION TIME (HOURS)


B
^{125}I-TETANUS TOXIN BOUND
(fmol/mg)
250
150
50
150
90
30
EVOKED ACh RELEASE
(Dpm x 10^{-3}/mg)
4
8
12
18
24
INCUBATION TIME (HOURS)


C
^{125}I-TETANUS TOXIN BOUND
(fmol/mg)
2500
1500
500
150
90
30
EVOKED ACh RELEASE
(Dpm x 10^{-3}/mg)
1
2
3
4
INCUBATION TIME (HOURS)


EVOKED ACh RELEASE
(dpm x 10 $^{-2}$ /mg protein)
100
75
50
25
37°→37°
37°→ 0°
0°→0°
INCUBATION CONDITIONS


B
EVOKED ACh RELEASE
(dpm x 10^{-2}/mg protein)
100
75
50
25
CONTROL
IBMX
ZAP
INCUBATION CONDITIONS


A
EVOKED cGMP LEVELS
(fmol x 10^{-1}/mg protein)
45
30
15
CONTROL
IBMX
ZAP
INCUBATION CONDITIONS

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